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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Antizyme is a small, labile protein central to the regulation of polyamines and a likely candidate to mediate polyamine depletion as cancer therapy. Antizyme exists in several different forms in cells that may reflect variable function or cellular localization. To sort out the origin, modification, function and localization of the various forms of antizyme, a cell line with an inducible AZ-1 construct that can only result in protein synthesized from the first start site was produced.

Western blot analysis showed that the mutated antizyme gene, when expressed in cells, produces two proteins distinct in molecular weight. One of the forms appears to be derived by proteolytic truncation of the N-terminus. These forms of antizyme behave as native antizyme with respect to activity in binding and inhibiting ODC, as well as down-regulating the polyamine transport system. Characterization of the N-terminal truncation of antizyme was attempted by various means. Unfortunately, a specific cleavage site could not be determined. These results suggest that native antizyme is post-translationally modified and this modification may play a role in the regulation of antizyme.

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Introduction

The polyamines are small, organic molecules that are essential for normal cell physiology and growth. Polyamine levels are tightly regulated in cells, and perturbations in this homeostasis are associated with disease, the most notable of which is cancer. Breast tumors have elevated levels of polyamines compared to healthy tissue and these elevated levels promote tumor progression, contribute to estrogen independence, and confer a more invasive phenotype in breast cancer cells. Elevated polyamine levels in breast cancer cells are achieved by an increase in both *de novo* synthesis as well as uptake from the gastrointestinal tract.

Antizyme is a small, scarce, labile protein central to the regulation of polyamines. This regulatory protein inhibits synthesis of polyamines through its interaction with ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine synthesis. In addition, antizyme acts to inhibit the uptake of polyamines from the extracellular environment. Since antizyme acts on both the synthesis and uptake of polyamines, it is a prime target to mediate polyamine depletion as cancer therapy. Unfortunately, very little is known about this regulatory molecule. Antizyme exists in several different forms in cells that may reflect variable function or cellular localization. Understanding antizyme form and function and its relation to the regulation of polyamine levels in both normal and diseased cells is critical in understanding tumor development and breast cancer.

The purpose of this study was to characterize the post-translational processing, cellular localization and activity of the forms of antizyme originating from the first start site of the AZ-1 gene. To this end, a cell line with an inducible AZ-1 construct that can only result in protein synthesized from the first start site was produced and studied.

Body

Specific Aim 1: Determine which antizyme forms originate from the first start site of the AZ-1 gene.

To sort out the origin, modification, function and localization of the various forms of antizyme, cell lines with inducible AZ-1 constructs that can only result in protein synthesized from the first start site were produced. Both constructs have the full AZ-1 mRNA coding sequence with the following alterations: 1) a mutation (one conservative [Met34 to Ser] and one non-conservative [Met34 to Arg]) at the second start site, eliminating the possibility of translation beginning there; 2) a 6-His tag and V-5 epitope at the C-terminus to distinguish inserted from native antizyme, since there are no antizyme-negative cells available to serve as host for transfection, and; 3) a T deletion to obviate the +1 frameshift necessary for translation of native antizyme, eliminating the polyamine requirement for expression. These AZ-1 constructs were made, cloned into the GeneSwitch system (Invitrogen) and confirmed by sequencing. The GeneSwitch system is a mammalian expression system that keeps inserted genes tightly repressed in the un-induced state. Cells were then stably transfected with the constructs and induced with Mifepristone.

As previously reported, both of these constructs produced in both Chinese hamster ovary (CHO) and rat hepatoma (HTC) cells resulted in two forms of antizyme. The size difference between the two mutated forms (37 and 32 KDa), about 5 KDa, is similar to that observed between the two major native antizyme forms (29.5 and 24 KDa).

In both cases the smaller form is the predominant band. Since it appears that the more prominent smaller antizyme state is formed by N-terminal proteolytic truncation in the mutated antizyme constructs, it is likely that this same processing may occur in the wild-type antizyme. Then, some, and perhaps most, of the 24 KDa antizyme form may be derived by proteolytic truncation of the 29.5 KDa form.

Although the discovery that the two major antizyme forms might both originate from the fist start site of AZ-1 was surprising and exciting, the physiological relevance was not clear. One factor relevant to the physiology of the mutated antizyme was that it was not known whether the tags had any effect on antizyme activity or localization. One of the first considerations, prior to further exploration of the nature of this apparent modification, was determination of the activity and localization of the protein produced from the constructs.

Specific Aim 2: Determine the activity of the antizyme forms produced.

As reported previously, antizyme produced from the constructs, both in rabbit reticulocyte lysate and transfected cells, showed normal activity in binding and inhibiting ODC. In addition to mediating feedback regulation of ODC, and thus, polyamine synthesis, antizyme also serves to control polyamine transport. To determine if the mutated antizyme was active in down-regulating polyamine transport into cells, 14-C spermidine transport experiments were conducted. Results of these experiments showed that the mutated antizyme displays normal antizyme activity in down-regulating polyamine transport.

Specific Aim 3: Determine the cellular localization of antizyme forms produced.

Cell fractionation by differential centrifugation was performed on transfected cells induced with Mifepristone and lysed by nitrogen cavitation. As previously reported, Western blot analysis on each fraction, using anti-antizyme antibody was performed to determine which fractions contained the mutated antizyme. As with native antizyme, mutated antizyme localized to every cell fraction. There was no distinction in localization with respect to form. It was expected that if the full-length protein, containing the putative mitochondrial translocation signal, were transported into the mitochondria and then the signal sequence was cleaved, the processed form would only be seen in the mitochondrial pellet. This was not seen, however approximately 20% of the mutated antizyme was found to be associated with the mitochondrial pellet.

Specific Aim 4: Purify enough antizyme to sequence the N-terminus of each form produced.

Since the construct antizyme behaves as native antizyme with respect to activity and localization, the apparent N-terminal truncation observed with the construct protein is likely to reflect a similar change occurring with native antizyme. In order to study this proteolytic processing, it was decided that identification of the cleavage site would provide clues as to what protease was responsible and why.

Since there was no apparent difference in processing, activity or localization in conservative vs. non-conservative constructs in both HTC and CHO cells, the highest producing clone was selected for purification purposes. Protein from this clone was purified using a metal-ion column made with Talon resin (Clontech). The concentrated

protein product was separated on SDS-PAGE and blotted to an Immobilon-PSQ membrane (Millipore). The processed, 32 KDa band was cut and submitted to commercial protein micro-sequencing facilities (University of Illinois and Midwest Analytical) for evaluation of the first seven amino acids. Unfortunately, after repeated attempts, no antizyme sequence was obtained. In the opinion of the sequencing facility, the protein's N-terminus is chemically blocked.

In eukaryotic cells, a blocked N-terminus is common (40-90% of all proteins). Common blocks include formyl, acetyl and pyroglutamyl moieties. For antizyme, the most likely common block is acetyl. Many amino acids can be acetylated, but the most common are serine and threonine. For this reason, unblocking attempts were made using trifluoroacetic acid treatment. Multiple protein bands were cut from the membrane, treated and sent for sequencing. Again, no sequence was obtained, which suggests that the blocking group on antizyme is not an acetyl-serine or threonine.

Upon analysis of the amino acid sequence extrapolated from the gene sequence, it was noted that antizyme has a four amino acid identity to S-adenosylmethionine decarboxylase (SAMdc) and this sequence, is where SAMdc is autocatalytically processed, resulting in an N-terminal pyruvyl-blocked protein. Since this four amino acid sequence is at a location in antizyme that could result in the processed antizyme form seen, it was concluded that antizyme might be processed and blocked in the same manner as SAMdc. Again, the same purification procedure was repeated and the protein was subjected to reductive amination, which unblocks proteins with N-terminal pyruvate moieties. No antizyme sequence was obtained, which suggests that the blocking group on antizyme is not a pyruvate.

Since all efforts to sequence the N-terminus of the 32 KDa form were unsuccessful, a second approach to identify the proteolytic cleavage sight was attempted. Theoretically, the mass of the 32 KDa band could be determined by mass spectroscopy, which would narrow down to a few amino acids where the cleavage site might be. As for sequencing, protein was purified and the 32 KDa antizyme band was cut from the membrane and subjected to Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectroscopy. MALDI has proven to be the most successful method to determine the molecular mass of large molecules like proteins. This analysis failed to identify any molecules of the anticipated mass, which indicates that either there was not enough protein or the protein adhered too well to the membrane.

A third approach to identify the site of cleavage was to mutate regions surrounding the estimated cleavage site. The assumption was that disrupting the amino acid sequence surrounding the cleavage site would prevent such cleavage. Thus three new constructs were made, with further mutations. Collectively, these mutations were 10 amino acids up- and downstream of the mutated second start site. These constructs were transfected into cells, under the control of the GeneSwitch system, and expressed. None of these mutations failed to process. These data suggest that the sequences mutated are not required for the observed modification.

Specific Aim 5: Confirm results with the use of AZ-1/GFP fusion proteins

To confirm results obtained by cellular fractionation, constructs of the modified AZ-1 gene, as discussed in Specific Aim 1 were made with a green fluorescent protein (GFP) tag in place of the V5 epitope. The plan was to use the fluorescent properties of

the GFP and resolution of laser scanning confocal microscopy (LSCM). Results of these experiments, contrary to cellular fractionations, showed that in individual cells, antizyme localizes mostly to the nucleus in some cells and in other cells it appears in a punctate peri-nuclear pattern.

Previous studies, performed by other groups, noted this same nuclear and nuclear-excluded pattern of localization for antizyme in fixed tissues. These groups concluded that the difference in antizyme localization was the result of differences in cell type. However, in these studies, the cells visualized were clones. Therefore, the difference between cells with nuclear versus nuclear-excluded localization must be some physiological difference (i.e. cell cycle event or apoptosis) that can be observed in cells of the same type. To determine what this might be, induced cells were synchronized by mitotic shake-off every hour for 24 hours and then visualized by LSCM. No apparent differences were noted at any time point, which suggests that the distinct localization pattern is not dependent on phase of the cell cycle.

To investigate specific localization of the AZ-1/GFP fusion protein in cells, visualization of organelles, using cell-permeable fluorescent dyes, was attempted. Since cell fractionation studies suggested that 20-30% of cellular antizyme is bound to or in the mitochondria, an attempt was made to visualize this interaction, using MitoTracker Orange. Results from these studies suggested that at least some cellular antizyme co-localizes with mitochondria.

Visualization of other organelles (i.e. nuclei) was attempted, using other organelle-specific dyes. Unfortunately, the limitations of the LSCM apparatus (lasers and filter sets) and the wide emission spectra of GFP prevented meaningful conclusions.

Key Research Accomplishments

- AZ-1 constructs with the second start site mutated were made, confirmed and transfected in CHO and HTC cells.
- Two forms of antizyme orginating from the first start site of AZ-1 constructs were identified as 37 KDa and 32 KDa bands, which correlate with the 29.5 KDa and 24 KDa forms of native antiyzme.
- Antizyme activity of the protein produced from the AZ-1 constructs was determined and found to be active in binding and inhibiting ODC.
- Antizyme activity of the protein produced from the AZ-1 constructs was determined and found to be active in inhibiting polyamine transport.
- Cellular localization of antizyme originating from the first start site of AZ-1 was determined to be comparable to that of wild-type antizyme.
- Cellular localization of AZ-1/GFP fusion protein was visualized in individual cells, using Laser Scanning Confocal Microscopy and showed two patterns of localization: nuclear and nuclear excluded.
- Partial co-localization of antizyme with mitochondria, in individual cells, was seen using AZ-1/GFP fusion protein and a cell-permeable mitochondrial dye.
- Although no specific site of cleavage could be obtained, the N-terminal truncation of antizyme results in a blocked N-terminus. Further, it appears that the 20 amino acids surrounding the second start site of antizyme are not necessary for the N-terminal processing.

Reportable Outcomes

Research

Presentations

- Poster. Sept. 2002. Era of Hope Department of Defense Breast Cancer Program Meeting, Orlando, FL, "Antizyme Activation in Chemotherapy and Chemoprevention"
- Poster. Feb. 2002. Office of Sponsored Projects Poster Exhibit, Northern Illinois University, DeKalb, IL, "Antizyme Activation in Chemotherapy and Chemoprevention"
- Poster. June 2001. Gordon Research Conference on Polyamines, New London, CT, "Antizyme Activation in Chemotherapy and Chemoprevention"
- Poster. April 2001. Biological Research Symposium, Northern Illinois University, DeKalb, IL, "Antizyme Forms Originating From the First Start Site of the AZ-1 Gene"
- Poster. Feb. 2001. Office of Sponsored Projects Poster Exhibit, Northern Illinois University, DeKalb, IL, "Antizyme Forms Originating From the First Start Site of the AZ-1 Gene"

Products

Cell lines, tissue, or serum repositories developed

- CHO and HTC cell lines containing an inducible AZ-1 gene with the second start site mutated were developed.
- A CHO cell line containing an inducible, mutated AZ-1/GFP fusion protein was developed.

Career Development

- March 2003. Successfully defended dissertation.
- April 2001. Phi Sigma Outstanding Graduate Research Award.

Conclusions

The AZ-1 gene produces two forms of antizyme from the first start site, one of which is derived by proteolytic processing. These forms of antizyme behave as native antizyme with respect to activity in binding and inhibiting ODC, inhibiting polyamine uptake, and cellular localization as determined by cellular fractionations. This proteolytic processing may impact its function and/or localization. Attempts were made to determine the cleavage site so that further characterization of the processing and its importance would be possible. Unfortunately, no specific site could be identified. LSCM analysis of localization of antizyme in AZ-1/GFP expressing cells showed that in individual cells antizyme localizes in either punctate peri-nuclear or nuclear pattern. Further, some antizyme appears to co-localize with mitochondria. Antizyme activity deficiency in certain aggressive tumors may be correlated with abnormalities in antizyme processing and cytolocalization observed in these studies.